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USE OF ACTIVE P40 CONJUGATES FOR NASAL DELIVERY

The present invention relates to the production of immunizing preparations which are effective in nasal administration. It thus relates to the use of carrier proteins which can improve the immune response to a hapten when the hapten/carrier protein conjugate is administered nasally.

The use of vaccine for oral or nasal delivery is thought to have a great influence on the eradication of pathogenic microorganisms. Specifically, any modification of a vaccine which allows it to be used with greater flexibility (heat-stability, distribution without syringes, etc.), would result in a more effective and more widely used vaccination. On the other hand, immunization via the mucous membrane pathways makes it possible to induce a local immunity which constitutes the first barrier against invasion by a microorganism.

Currently, the oral vaccines which are on the market only concern attenuated or recombined live vectors:

- tetravalent oral vaccine against polio,
- oral vaccine against typhoid fever.

Approaches for nasal or oral vaccination are already described in the literature.

Tests have thus been carried out on mucosal administrations of PspA, which corresponds to the surface protein A of Pneumococcus (Briles D.E., patent EP 0,682,950), on hemaglutinin filaments (Capron A., patent FR 2,718,750; Kimura A., patent EP 0,471,177; Shahin R.D., US patent 7532327), on a fragment of the tetanus toxin (Dougan G., patent WO 93/21950) and on cholera toxin B (CTB).

A protein of the external membrane of Neisseria *meningitidis* is used, mixed with the hapten as an adjuvant for a nasal immunization (Van de Verg L.L., Infection and immunity, 1996, 64: 5263-5268).

Unexpectedly, the Applicant has now found that a membrane protein originating from another bacterium makes it possible, when it is administered nasally together with an antigen, to induce an immune response of satisfactory strength and quality for the production of a vaccine.

For this reason, the subject of the present invention is the use of at least one fragment of an enterobacterium membrane protein OmpA for preparing a pharmaceutical composition intended to be administered nasally, to improve the immunity of a mammal with respect to an antigen or to a hapten.

In the present invention, the term "OmpA" is intended to refer to the type A proteins of the external membrane (OmpA for Outer membrane protein A).

A subject of the invention is also the use of at least one fragment of a membrane protein of Klebsiella for preparing a pharmaceutical composition intended to be administered nasally, to improve the immunity of a mammal with respect to an antigen or to a hapten.

Preferably, the membrane protein is an OmpA protein of Klebsiella *pneumoniae*.

Advantageously, said fragment of the enterobacterium membrane protein OmpA or of the Klebsiella membrane protein according to the invention is obtained by recombinant process.

Very advantageously, said membrane protein or its fragment, obtained by recombinant process, is, after extraction, renatured in the presence of detergent chosen from Zwittergent 3-14, Zwittergent 3-12 and octylglycopyranoside, preferably in the presence of Zwittergent 3-14 at a concentration of between 0.05% and 2% (w/v), very preferably at a concentration close to 0.1%.

Application WO 96/14415 has shown that the major membrane protein of Klebsiella *pneumoniae*, which is the OmpA named P40, coupled to peptide subunit antigens is very immunogenic via the systemic route.

The recombinant P40 protein, expressed in E.Coli in the form of inclusion bodies, is named rP40.

In the context of the present invention, a particularly suitable protein comprises the sequence SEQ ID No 1.

The Applicant has demonstrated that an anti-P40 antibody response is found in all adults, the enterobacterium Klebsiella *pneumoniae* being a very widespread pathogen. This sensitization favors an increase in the antibody response directed against an antigen or a hapten which is administered while coupled to the carrier protein P40. The administration is carried out nasally in the absence of adjuvant.

Said antigen or hapten according to the invention can be chosen from the group comprising proteins, peptides, polysaccharides, oligosaccharides and nucleic acids. Advantageously, it is of bacterial or viral origin.

The present invention is thus suitable for preparing vaccine directed against any microorganism responsible for pathologies of the airways, such as for example microorganisms chosen from RSV, parainfluenzae virus (PIV), influenza virus, hantavirus, streptococci, pneumococci and meningococci.

The antigen or hapten according to the invention will comprise at least one fragment of said microorganism, such as a protein fragment, which persons skilled in the art will know how to determine for its capacity to confer the desired immunity, by standard techniques such as those described in the examples below.

In particular, the present invention is suitable for preparing vaccine directed against RSV (or respiratory syncytial virus), in particular human or bovine RSV. In this case, the antigen or hapten according to the invention comprises at least one protein fragment of the virus RSV, and in particular at least one fragment of the protein G of the RSV.

The sequences of such fragments have in particular been described in application WO 95/27787.

Preferably, said protein fragments of the virus RSV are chosen from the fragments having the sequences SEQ ID No 2 to SEQ ID No 74 as amino acid sequences.

Sequences which are suitable for preparing a vaccine according to the invention are the sequences SEQ ID No 2 to SEQ ID No 74.

The chemical conjugates derived from the coupling of peptides to at least one fragment of a membrane protein of Klebsiella, such as rP40, give good results, and an evaluation of the immune response shows very strong antibody responses against these peptides after presensitization with Klebsiella *pneumoniae*.

Advantageously, the protein fragment originating from enterobacteria membrane protein OmpA or from membrane protein of Klebsiella is covalently coupled to the antigen or hapten, such as a protein fragment of the RSV.

The invention also comprises the use of at least one fragment of an enterobacteria membrane protein OmpA or of a membrane protein of Klebsiella according to the invention, characterized in that said fragment is covalently coupled to said antigen or hapten.

According to the invention, it is possible to introduce one or more bonding elements, in particular amino acids, to facilitate the coupling reactions between the fragment of membrane protein and the antigen or hapten.

The covalent coupling of the antigen or hapten according to the invention can be carried out at the N- or C-terminal end of the fragment of the membrane protein according to the invention. The bifunctional reagents which allow this coupling can be determined as a function of the end of the fragment of the membrane protein which is chosen to perform the coupling, and of the nature of the antigen or hapten to be coupled.

These coupling techniques are well known to persons skilled in the art.

The conjugates derived from the coupling of peptides to at least one fragment of an enterobacteria membrane protein OmpA or of a membrane protein of Klebsiella can be prepared by genetic recombination. The hybrid protein (conjugate) can in fact be produced by recombinant DNA techniques, by insertion or addition of a sequence encoding the antigenic or hapten peptide(s) into or to the DNA sequence encoding the fragment of membrane protein. These techniques for preparing hybrid protein by genetic recombination are well known to persons skilled in the art (cf. for example S.C. MAKRIDES, 1996, Microbiologicals Reviews, 60, 3, 512-538) and will not be developed in the present description.

Thus, the invention also comprises the use, according to the invention, characterized in that the hybrid protein, obtained after coupling between the fragment of a membrane protein and the antigen or hapten, protein in nature, is prepared by genetic recombination.

The Applicant has also shown that, in the absence of sensitization to Klebsiella *pneumoniae*, the nasal administration of a hapten coupled to at least one fragment of a membrane protein, such as the rP40 protein, in the absence of adjuvant, induced an anti-hapten antibody response.

The invention relates to the use, according to the invention, characterized in that the pharmaceutical composition contains a fragment of a membrane protein coupled to an antigen or hapten according to the invention, or a transformed host cell which is capable of expressing a hybrid recombinant protein containing a fragment of membrane protein coupled to the antigen or hapten according to the invention, in particular in the absence of adjuvant. Among the transformed host cells which are capable of expressing said hybrid protein, gram-negative bacteria such as Klebsiella *pneumoniae*,

Escherichia *coli* type K12 currently used in fermentation, or *E. coli* transformed with an expression vector plasmid containing a strong promoter such as the operon of the tryptophan promoter (trp) are preferred. Also preferred are gram-positive bacteria such as the nonpathogenic staphylococci, *S. carnosus* and *S. xylosus*, since these bacteria do not produce any LPS (lipopolysaccharides) at the membrane surface. These staphylococci can be transfected with expression vectors containing promoters such as trp, or the secretion signal of lipase or even the secretion signal of protein A, or alternatively the signal of the promoter of OmpA of Klebsiella *pneumoniae*.

Finally, the invention relates to a method for preparing a protein or one of its fragments by the recombinant pathway, characterized in that the protein or its fragment is, after extraction, renatured in the presence of a solution containing a detergent chosen from Zwittergent 3-14, Zwittergent 3-12 and octylglucopyranoside, and in that said recombinant protein is not interferon β.

Preferably, said protein is an enterobacterium membrane protein, in particular of OmpA type. Very preferably, said protein is an OmpA of Klebsiella *pneumoniae*.

In the method according to the invention, the Zwittergent 3-14 will be preferably at a concentration of between 0.05% and 2%, more preferably close to 0.1%.

The following examples are intended to illustrate the invention without in any way limiting the scope thereof.

In these examples, reference will be made to the following figures:

Figures 1A and 1B: Analysis by SDS-PAGE electrophoresis of the rP40 protein after purification.

Figure 1A: detection with Coomassie blue

- lane 1: batch 1, 2 µg
- lane 2: batch 1, 10 µg
- lane 3: batch 2, 2 µg

- lane 4: batch 2, 10 µg
- lane 5: batch 3, 2 µg
- lane 6: batch 3, 10 µg

Figure 1B: immunoblot and detection with the aid of an anti-P40 rabbit polyclonal serum

- std: molecular mass standard
- lane 1: denatured rP40, 100 ng
- lane 2: native rP40, 100 ng.

Figure 2: Division of the patients according to the O.D. (Optical Density) corresponding to the anti-P40 antibodies, measured by ELISA.

Figure 3: Anti-G1' antibody response.

Figure 4: Anti-rP40 antibody response.

Figure 5: Anti-G1' IgA-type antibody response.

Figure 6: Isotyping of the anti-G1' immunoglobulins obtained in secondary response.

Figure 7: Isotyping of the anti-G1' immunoglobulins obtained in tertiary response.

Figure 8: Anti-G1' total IgG-type serum antibody response.

Figure 9: Isotyping of the serum anti-G1' immunoglobulins after three immunizations.

Figure 10: Isotyping of the anti-G1' immunoglobulins from broncho-alveolar washes after three immunizations.

Example 1: Cloning of rP40

Cloning of the rP40 gene:

The gene encoding rP40 was obtained by amplification by PCR (Polymerase Chain Reaction) from the chromosomal DNA of the Klebsiella *pneumoniae* IP I145 strain (described in patent WO 96/14415). After identification by DNA sequencing, the fragment corresponding to rP40 is cloned into diverse expression vectors, in particular the one under the control of the trp operon promoter, upstream of 9 amino acids of the leader peptide (MKAIFVLNA). The peptide sequence of rP40 is represented in the sequence listing by the sequence SEQ ID No 1. In various *E.coli* K12 strains, the rP40 protein is produced in the form of inclusion

bodies with a considerable yield (> 10%, g proteins/g of biosolids).

Fermentation of rP40 fusion proteins:

E. coli K12 transformed with the plasmid pvaLP40 is inoculated in an Erlenmeyer flask containing 250 ml of TSB (Tryptic Soy Broth, Difco) medium containing ampicillin (100 µg/ml, Sigma) and tetracycline (8 µg/ml, Sigma). This is incubated for 16 hours at T° = 37°C with stirring. 200 ml of this culture are inoculated in a fermenter (CHEMAP CF3000, ALFA LAVAL) containing 2 liters of culture medium. The medium contains (g/l): glycerol, 5; ammonium sulfate, 2.6; potassium dihydrogen phosphate, 3; dipotassium hydrogen phosphate, 2; sodium citrate, 0.5; yeast extract, 1; ampicillin, 0.1; tetracycline 0.008; thiamine, 0.07; magnesium sulfate, 1 and 1 ml/l of trace element solution and 0.65 ml/l of vitamin solution. The parameters which are controlled during the fermentation are: pH, stirring, temperature, degree of oxygenation, supply of combined sources (glycerol or glucose). The pH is regulated at 7.0. The temperature is fixed at 37°C. The growth is controlled by supplying with glycerol (87%) at a constant flow rate (12 ml/h) so as to maintain the dissolved oxygen tension signal at 30%. When the turbidity of the culture (measured at 580 nm) reaches the value of 80 (after approximately 24 hours of culture), protein production is induced by adding indole acrylic acid (IAA) to a final concentration of 25 mg/l. Approximately 4 hours after induction, the cells are harvested by centrifugation. The amount of biomass obtained is approximately 200 g, expressed as wet biomass.

Example 2: Extraction and purification of rP40

Materials and methods

Extraction of rP40

After centrifugation of the culture medium (4000 rpm, 10 min, 4°C), the cells are resuspended in a 25 mM Tris-HCl buffer, pH 8.5. A treatment with

lysozyme (0.5 g/l, 1 hour/room temperature/gentle stirring) allows the release of the inclusion bodies.

The pellet of inclusion bodies obtained by centrifugation (25 min at 10,000 g at 4°C) is taken up in a 25 mM Tris-HCl buffer, pH 8.5, containing 5 mM $MgCl_2$, and then centrifuged (15 min at 10,000 g).

The denaturation of the protein is obtained by incubating the inclusion bodies at 37°C for 2 hours in a 25 mM Tris-HCl buffer, pH 8.5, containing 7 M urea (denaturing agent) and 10 mM dithiothreitol (reduction of disulfide bridges). A centrifugation (15 min at 10,000 g) makes it possible to remove the insoluble portion of the inclusion bodies.

After dilution with 13 volumes of a 25 mM Tris-HCl buffer, pH 8.5, containing NaCl (8.76 g/l) and Zwittergent 3-14 (0.1%, w/v), the mixture is left to stand overnight at room temperature with stirring, in contact with the air (renaturation of the protein by dilution and reoxidation of the disulfide bridges).

Purification of the rP40 protein

Anion exchange chromatography step.

After another centrifugation, the sample is dialyzed against a 25 mM Tris-HCl buffer, pH 8.5, containing 0.1% Zwittergent 3-14 (100 volumes of buffer) overnight at 4°C.

The dialyzate is loaded onto a column containing a support of strong anion exchanger type (Biorad Macro Prep High Q gel), which is equilibrated in the buffer described above at a linear flow rate of 15 cm/h. The proteins are detected at 280 nm. The rP40 protein is eluted, with a linear flow rate of 60 cm/h for an NaCl concentration of 0.6 M, in the 25 mM Tris/HCl buffer, pH 8.5; 0.1% Zwittergent 3-14.

Cation exchange chromatography step.

The fractions containing the rP40 protein are pooled and concentrated by ultrafiltration with the aid of an Amicon cell system with stirring used with a Diaflo membrane of type YM10 (cutoff threshold 10 kDa) for volumes of about 100 ml, or with the aid of a

Millipore Minitan tangential flow filtration system used with membrane plates having a cutoff threshold of 10 kDa, for larger volumes. The fraction thus concentrated is dialyzed overnight at 4°C against a 20 mM citrate buffer, pH 3.0, containing 0.1% of Zwittergent 3-14.

The dialysate is loaded onto a column containing a support of strong cation exchanger type (Biorad Macro Prep High S gel), which is equilibrated in the 20 mM citrate buffer, pH 3.0, containing 0.1% of Zwittergent 3-14. The rP40 protein is eluted (rate 61 cm/h) for a 0.7 M NaCl concentration. The fractions containing the rP40 are pooled and concentrated as described above.

Results

Starting from a 1 liter culture, one denaturation/renaturation cycle makes it possible to obtain 300 mg of protein (estimation by assay according to the Lowry method). 75 mg of rP40 are purified after the two chromatographic steps.

As above, the rP40 protein is concentrated after purification in order to attain a final concentration of between 5 and 10 mg/ml. The electrophoretic profiles show a degree of purity of about 95% (Figure 1A). After immunoblot, the protein is specifically recognized by an anti-natural P40 monoclonal antibody obtained in mice (Figure 1B).

The condition of the protein is monitored by SDS-PAGE. Depending on its form, denatured or native, the P40 protein extracted from the membrane of Klebsiella *pneumoniae* has a characteristic electrophoretic behavior (migration). The native form (β-sheet structure) in fact has a lower molecular mass than the denatured form (α-helix structure) under the action of a denaturing agent, such as urea or guanidine hydrochloride, or with heating to 100°C in the presence of SDS (Figure 1B). The rP40 protein is not correctly renatured at the end of renaturation, regardless of whether this is carried out in the presence or absence

of 0.1% (w/v) Zwittergent 3-14. Conversely, total renaturation is obtained after dialysis against a 25 mM Tris/HCl buffer, pH 8.5, containing 0.1% (w/v) Zwittergent 3-14. However, it should be noted that this renaturation is only obtained when the dilution step and the treatment at room temperature are themselves carried out in the presence of Zwittergent 3-14 (negative results in the absence of detergent).

Example 3: Coupling of the G1' peptide to rP40

Materials and methods

The G1' peptide is a synthetic peptide of 15 amino acids, the sequence of which is as follows (SEQ ID No 74):

$N\text{-}_1SIDSNNPTOWAISKC_{15}\text{-}C$

Without the Cys (cysteine) residue added in the C-terminal position, this peptide (portion 1-14) corresponds to portion 174-187 of the protein G of the respiratory syncytial virus, and has, with respect to the native peptide, two major modifications which are:

- the replacement of the Cys residue at position 13 with a Ser (serine) residue,
- the replacement at positions 3 and 9 of the Cys residues, which form a disulfide bridge, with, respectively, Asp (aspartic acid) and Orn (ornithine) residues which form a lactam-type bridge.

These modifications are introduced for the purpose of removing the Cys residues of the native peptide in order to be able to carry out a one-to-one coupling of the latter to the protein via the Cys residue introduced in the C-terminal position, while at the same time maintaining the structure of the peptide with the aid of the introduction of a lactam bridge.

The coupling of the peptide to the protein is carried out using the BHA or bromo-N-hydroxysuccinimide acetate reagent (Svenson et al., 1990, Proc. Natl. Acad. Sci. USA 87, 1347, Bernatowicz and Matsueda, 1986, Anal. Biochem. 155, 95). This heterobifunctional reagent allows activation of the Lys (lysine) residues of the protein by bromoacetylation, and then coupling

of the peptide via the free thiol group carried by the Cys residue.

Firstly, the rP40 protein is activated with the BHA. The rP40 is dialyzed against a 0.1 M phosphate buffer, pH 7, containing 0.1% Zwittergent 3-14, for 24 hours at +4°C. After dialysis, the concentration is adjusted to 5 mg/ml with the aid of the same buffer, before adding BHA in a proportion of 1.2 mg (50 µl)/mg of rP40.

The whole is placed in the dark for one hour with stirring and at room temperature.

The activated rP40 is then desalified by gel filtration chromatography (elution with the abovementioned buffer). The fractions containing the bromoacetylated protein are pooled.

For the coupling, the peptide (10 mg/ml in 0.1 M phosphate buffer, pH 7, containing 0.1% Zwittergent 3-14) is added to the activated protein in a proportion of 0.4 mg/mg of protein. After saturation under a nitrogen stream, the tube is again placed in the dark for 2 hours with stirring and at room temperature.

The unbound peptide can be removed with the aid of a dialysis step or of molecular sieve chromatography.

Results

The conjugate obtained is characterized by protein assay (BCA or LOWRY method) and by SDS-PAGE electrophoresis. The degree of coupling of the peptide to the protein is estimated by carboxymethylcysteine residue assay: the assaying of the amino acids released by hydrolysis (6N HCl) is performed by HPLC after derivatization with the aid of PITC (Pico-Tag method, Waters).

The degree of coupling determined by this method is approximately 10 G1' peptides/mole of rP40.

Example 4: Natural immunity in adults

Human sera derived from a clinical study are analyzed by ELISA assay in order to determine the presence of anti-P40 antibodies.

The results are represented in Figure 2.

Among 113 sera tested after 400-fold dilution, 110 sera give a colorimetric signal revealing the anti-P40 IgGs. There are circulating anti-P40 antibodies in all the patients, with levels which are more or less high depending on the patient under consideration.

Example 5: Anti-G1' antibody response after sensitizations and frequent immunizations

BALB/c mice were or were not sensitized twice with a Klebsiella *pneumoniae* I145 strain, in order to reproduce the seropositivity found in humans. The mice are subsequently immunized nasally in the absence of adjuvant 7 days after the sensitization. This immunization is carried out with a small amount of antigen, the mice receiving 10 µg of G1' equivalent coupled to rP40. The mice receive a booster 10 and 20 days after the first immunization. A retro-orbital sinus puncture is performed on the mice 9 days after the first immunization and 10 days after each booster (secondary and tertiary responses). The serum anti-G1' (Figure 3) and anti-carrier (Figure 4) antibodies are assayed by the ELISA method.

5.1 Assaying of anti-G1' serum IgGs

The results are represented in Figure 3.

In the primary response, the mice presensitized with Klebsiella *pneumoniae* and immunized with rP40-G1' are the only ones to produce anti-G1' antibodies.

The level of anti-G1' antibodies found in the mice presensitized with Klebsiella *pneumoniae* and immunized with rP40-G1' is increased after a second immunization. In the absence of presensitization, a second immunization in the presence of the rP40-G1' conjugates induces an anti-G1' antibody response.

After three immunizations, the anti-G1' antibody response is increased in the presensitized and non-presensitized mice.

5.2 Assaying of anti-rP40 serum IgGs

The results are represented in Figure 4.

The anti-P40 antibody response shows that the mice were sensitized to Klebsiella *pneumoniae* in identical manner whatever the batch under consideration.

The immunization in the presence of rP40-G1' conjugates slightly increases the anti-rP40 antibody response.

5.3 Assaying of anti-G1' serum IgAs

Secondly, we assayed the serum IgA-type anti-G1' antibody response: immunoglobulin characteristic of immunizations carried out via the mucous membrane (nasal or oral) pathways.

The results are represented in Figure 5.

After a single immunization, IgAs are not detected. After two immunizations, anti-G1' IgAs are detected essentially in mice presensitized to Klebsiella *pneumoniae* and immunized with rP40-G1'. This response is increased by the third immunization. In the absence of sensitization, anti-G1' IgAs are detected in mice after two immunizations with rP40-G1' conjugates. This level of IgA is increased by the third immunization.

5.4 Isotyping of anti-G1' serum immunoglobulins

Two types of response can be observed, Th1 and Th2. These responses differ by the profile of cytokines produced and by their functions in the immune response. IgG1s are characteristic of a response of type Th2, and IgG2as are characteristic of a Th1 response.

A mixed Th1 and Th2 response profile is found only in the mice immunized with the rP40-G1' conjugates, whether or not they are presensitized with Klebsiella *pneumoniae* (Figure 6).

After three immunizations (Figure 7), the profile remains mixed in the mice immunized with the rP40-G1' conjugates.

Example 6: Anti-G1' antibody response after sensitizations and infrequent immunizations.

With respect to the above protocol, the first immunization is separated from the final sensitization by a period of 3 weeks instead of one week. The anti-G1' antibodies are assayed in the sera, and, in the tertiary response, in broncho-alveolar washes, by the ELISA method.

6.1 Assaying of anti-G1' serum IgGs

As seen in Figure 8, 7 days after the first immunization, anti-G1' serum antibodies of type total IgG are detected in the mice presensitized to Klebsiella *pneumoniae* and immunized in the presence of the rP40-G1' conjugates. This antibody response is increased by the two other immunizations.

6.2 Isotyping of serum immunoglobulins

The results are represented in Figure 9.

In this case, we also observe a mixed response, we obtain in fact the same titer for IgG1 as for IgG2a (Figure 9). In addition, a high level of IgA is found in the mice presensitized to Klebsiella *pneumoniae* and immunized three weeks later in the presence of the rP40-G1' conjugates.

6.3 Isotyping of immunoglobulins from broncho-alveolar washes

In the broncho-alveolar washes, the 4 types of immunoglobulin are found only in the mice sensitized to Klebsiella *pneumoniae* and immunized 3 times in the presence of the rP40-G1' conjugates (Figure 10).